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Invention: PLASMIDS FROM CORYNEBACTERIUM GLUTAMICUM AND USE THEREOF

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SPECIFICATION

Plasmids from Corynebacterium glutamicum and use thereof

This application claims priority from German Application No. DE 199 53 206.0, filed on November 5, 1999, the subject matter of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention provides the novel plasmids pTET3 and pCRY4 and the use thereof for the production of vector plasmids.

2. Background Information

Naturally occurring plasmids and plasmid vectors produced therefrom are vital to the improvement of the production characteristics of coryneform bacteria. Constructing plasmid vectors for this group of industrially significant bacteria is substantially based on cryptic plasmids, which are provided with suitable selection markers capable of functioning in *Corynebacteria* or *Brevibacteria* (U.S. Pat. No. 5,158,891 and U.S. Pat. No. 4,500,640). These plasmid vectors may be used for cloning and amplifying genes which are involved in the production of L-amino acids, vitamins or nucleotides. Expression of these particular genes may have a positive influence on the production of the desired substances. Thus, for example, cloning a DNA fragment which encodes a protein for a lysine exporter resulted in an improvement in the fermentative production of L-lysine with *Corynebacterium glutamicum* strain MH20-22B (DE-A 19548222).

In contrast with the known and equally industrially significant bacterium *Escherichia coli*, only a limited number of natural plasmids and suitable selection markers are available for developing cloning and expression vectors

for *Corynebacteria* and *Brevibacteria*. Many plasmids known by different names prove to be identical on more detailed analysis of their genetic organisation. These plasmid isolates have thus been classed in two groups (Sonnen et al., Gene 107, 69-74 (1991)).

The pBL1 group includes the plasmids pAM286 from *Corynebacterium glutamicum* AJ11560 (EP-A 0093611), pAM330 from *Brevibacterium lactofermentum* ATCC13869 (Miwa et al., Agricultural and Biological Chemistry 48, 2901-2903 (1984)), pX18 from *Brevibacterium lactofermentum* ATCC21086 (Yeh et al, Gene 47, 301-308 (1986)) and pBL1 from Brevibacterium lactofermentum ATCC21798 (Santamaria et al., Journal of General Microbiology 130, 2237-2246 (1984)).

The pHM1519 group comprises plasmids pCG1 from *Corynebacterium glutamicum* ATCC31808 (U.S. Pat. No. 4,617,267), pHM1519 from *Corynebacterium glutamicum* ATCC13058 (Miwa et al., Agricultural and Biological Chemistry 48, 2901-2903 (1984)), pSR1 from *Corynebacterium glutamicum* ATCC19223 (Yoshihama et al., Journal of Bacteriology 162, 591-597 (1985)) and pRN3.1 from *Corynebacterium glutamicum* ATCC39269 (U.S. Pat. No. 4,559,308).

In addition to members of these two groups of plasmids, the cryptic plasmids pCG2 from *Corynebacterium glutamicum* ATCC31832 (U.S. Pat. No. 4,489,160) and pAG3 from *Corynebacterium melassecola* 22220 (U.S. Pat. No. 5,158,891) have also been isolated.

The only selection systems which have hitherto been available are two antibiotic resistance markers which were identified on the streptomycin/spectinomycin resistance plasmid pCG4 from *Corynebacterium glutamicum* ATCC31830 (U.S. Pat. No. 4,489,160) and on the tetracycline

resistance plasmid pAG1 from *Corynebacterium melassecola* 22243 (U.S. Pat. No. 5,158,891). Plasmid pCG4 also bears the sulI gene which imparts sulfamethoxazole resistance, the sequence of which gene was determined by Nesvera et al. (FEMS Microbiology Letters 169, 391-395 (1998)).

If strains which produce amino acids, vitamins or nucleotides are to be rapidly investigated and improved, it is important to have plasmid vectors which are mutually compatible and are sufficiently stable.

It is known from the prior art that pHM1519 plasmid derivatives and pBL1 plasmid derivatives may coexist. It is furthermore known that the plasmids pGA1 and pGA2 described in U.S. Pat. No. 5,175,108 are compatible.

Plasmid vectors having high, moderate or low copy numbers so that expression of the gene under consideration may be graduated are also of significance. Most known plasmids have a high copy number. Only the plasmid pGA2 described in U.S. Pat. No. 5,175,108 is known to have a low copy number.

The widely used plasmid vectors are composed of components originating from the species *C. glutamicum* and components from another species of bacteria, typically *E. coli*. This method introduces foreign DNA into the species *C. glutamicum*. Functional plasmid vectors with a graduated copy number which contain only endogenous DNA and thus meet the criteria of self cloning are not known in specialist circles.

SUMMARY OF THE INVENTION

Object of the invention

It is an object of the invention to provide novel plasmids that are suitable for constructing plasmid vectors having

improved characteristics for coryneform bacteria which produce amino acids, vitamins and nucleotides.

Description of the invention

Amino acids, vitamins and nucleotides are used in animal nutrition, in the food industry, in the pharmaceuticals industry and in human medicine. These substances are produced with strains of coryneform bacteria. Production characteristics are improved by amplifying suitable genes by means of plasmid vectors. There is accordingly general interest in providing novel plasmid vectors having improved characteristics.

The present invention provides the mutually compatible plasmids pTET3 and pCRY4, isolated from the strain of *Corynebacterium glutamicum* deposited under DSM number 5816, wherein

1.1 plasmid pTET3 is characterised by a length of ~ 27.8 kbp and the restriction map shown in Figure 1, and an antibiotic resistance region and

1.2 plasmid pCRY4 is characterised by a length of ~ 48 kbp and the restriction map shown in Figure 2.

The present invention also provides composite plasmids of pTET3 and pCRY4 capable of autonomous replication in coryneform bacteria, said plasmids containing

2.1 a part or the entire quantity of the nucleotide sequences

2.2 at least one DNA replication region derived from one of the plasmids pTET3 or pCRY4

2.3 optionally a gene fragment which is derived from a plasmid which can multiply in *E. coli*, *B. subtilis* or *Streptomyces* and

2.4 at least one region for expressing active substance resistance, preferably from the plasmid pTET3.

The present invention also provides composite plasmids which contain at least part of the active substance resistance(s) and pGA1 and/or pGA2 from the novel plasmids according to the invention.

The novel plasmid pTET3, the restriction map of which is shown in Figure 1, has

1. a replication region comprising the nucleotide sequence shown in SEQ ID NO:1 and
2. an antibiotic resistance region consisting of a tetA gene imparting tetracycline resistance and an aadA gene imparting streptomycin and spectinomycin resistance, shown in SEQ ID NO:6.

The novel plasmid pCRY4, the restriction map of which is shown in Figure 2, has a replication region comprising the nucleotide sequence shown in SEQ ID NO:4.

The present invention also provides the production of amino acids, vitamins and nucleotides using plasmid vectors (composite plasmids) which contain pTET3 and pCRY4 and optionally pGA1 or pGA2 nucleotide sequences.

Corynebacterium glutamicum LP-6, which was deposited as DSM5816 in the context of EP-B 0 472 869, contains the novel plasmids pTET3 and pCRY4 in addition to the plasmids pGA1 and pGA2 described therein. The storage period for DSM5816 has been extended pursuant to rule 9.1 of the Budapest Treaty.

Plasmids pTET3 and pCRY4 are prepared by culturing strain LP-6 in a conventional medium, such as for example brain-heart bouillon or Luria-Bertani medium. The cells were harvested by centrifugation, treated with lysozyme and digested by the alkaline lysis method. The DNA is then purified by anion exchange chromatography on silica gel particles, precipitated with ethanol or isopropanol and then resuspended in H_2O. Complete systems for isolating plasmid DNA are commercially available as "kits". One example of such a kit is the "NucleoBond Plasmid Kit" from Clonetech Laboratories GmbH. The person skilled in the art will find detailed instructions relating to the use of this kit in the manual "NucleoBond Nucleic Acid Purification Kits and Cartridges, User Manual (PT3167-1)" from Clonetech Laboratories GmbH (Heidelberg, Germany, 1997). Plasmids pTET3 and pCRY4 are revealed as plasmid bands by separating the total plasmid DNA obtained in this manner by agarose gel electrophoresis and staining with ethidium bromide. DNA from the plasmid pTET3 and DNA from the plasmid pCRY4 may then be isolated from the agarose gel. To this end, the agarose gel containing the plasmid DNA is combined with a chaotropic reagent, the plasmid DNA present in the resultant solution is bound onto the surface of glass or silica gel particles and then eluted back out from this matrix. The person skilled in the art will find detailed instructions relating to this process in the manual "QIAEX II Handbook for DNA Extraction from Agarose Gels" from Qiagen GmbH (Hilden, Germany, 1997). In this manner, it is possible to prepare pTET3 DNA and pCRY4 DNA in pure form.

DNA of the plasmid to be investigated is treated with restriction enzymes individually or in combination as described by Roberts et al. (Nucleic Acids Research 27, 312-313 (1999)). The resultant DNA fragments are separated by agarose gel electrophoresis and the restriction sites

assigned. The person skilled in the art will find instructions in this connection, for example, in Rodriguez and Tait "Recombinant DNA Techniques: An Introduction" (Addison-Wesley Publishing Company, London, 1983) or in "Guide to Molecular Cloning Techniques" edited by Berger and Kimmel (Methods in Enzymology, Vol. 152, Academic Press, London, 1987). In this manner, the length of the plasmid may be determined or the restriction map plotted. Plasmid pTET3 has a length of approximately 27.8 kbp and is shown in Figure 1. Plasmid pCRY4 has a length of approximately 48 kbp and is shown in Figure 2.

Plasmids pTET3 and pCRY4 have a moderate or low copy number. By virtue of this property, they advantageously complement the range of known plasmids for *Corynebacterium*. Instructions relating to determining copy number may be found, for example, in Miwa et al. (Agricultural and Biological Chemistry 48, 2901-2903 (1984)) and Vohradsky et al. (Electrophoresis 13, 601-612 (1993)).

In order to ensure simple handling of plasmids pTET3 and pCRY4, the DNA region responsible for replication on each plasmid is determined. Known plasmid vectors of *Escherichia coli* such as for example pK18 (Pridmore, Gene 56, 309-312 (1987)), pK18mob2 (Tauch et al., Plasmid 40, 126-139 (1998)) or pCR2.1 (Invitrogen BV, Groningen, Netherlands), which cannot replicate in coryneform bacteria, but the resistance gene of which is expressed, are used for this purpose. DNA from plasmids pTET3 and pCRY4 is isolated and treated with restriction enzymes. Individual DNA fragments obtained in this manner may optionally in turn be isolated. The DNA of the plasmid vectors used is treated with the same restriction enzymes or such enzymes that produce compatible ends. The resultant DNA molecules are mixed and treated with T4 DNA ligase. These "cloning" techniques were

known in the prior art and are described in detail in, for example, Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). After transforming a coryneform host, for example *Corynebacterium glutamicum*, with the ligation mixture and selecting for the resistance gene of the *E. coli* plasmid vector used, transformants are obtained. Instructions relating to the transformation of coryneform bacteria may be found, for example, in Thierbach et al. (Applied and Environmental Microbiology 29, 356-362 (1988)), in Liebl et al. (FEMS Microbiology Letters 65, 299-304 (1989)) or in Dunican et al. (Bio/Technology 7, 1067-1070 (1989)). The plasmid DNA of these transformants contains DNA segments of pTET3 or pCRY4, which impart the ability to replicate in coryneform bacteria. Examples of these are:

- plasmid pTET3-Rep, which consists of the *E. coli* plasmid pK18mob2 and the replication region of plasmid pTET3 (Figure 3), and

- plasmid pCRY4-Rep, which consists of the *E. coli* plasmid pK18mob2 and the replication region of plasmid pCRY4 (Figure 4).

The sections of DNA characterised in this manner are then in turn subcloned into usual vectors suitable for DNA sequencing. Examples of such vectors suitable for DNA sequencing are, for example, the plasmids pGEM-5zf(-) or pGEM-5zf(+) from Promega Corporation (Promega Protocols and Application Guide, Second Edition, 1991, part number Y981, Promega Corporation, Madison, WI, USA), plasmid pUC19 (Yanish-Perron et al., Gene 33, 103-119 (1985)) or plasmid pK18 (Pridmore, Gene 56, 309-312 (1987)).

DNA sequencing methods are described *inter alia* in Sanger et al. (Proceedings of the National Academy of Sciences of

the United States of America USA, 74, 5463-5467, 1977) and in Zimmermann et al. (Nucleic Acids Research 18, 1067 (1990)).

The resultant DNA sequences may then be investigated using known algorithms or sequence analysis programs, for example the "STADEN computer software package" (Molecular Biotechnology 5, 233-241 (1996)), Butler's GCG program (Methods of Biochemical Analysis 39, 74-97 (1998)), Pearson & Lipman's FASTA algorithm (Proceedings of the National Academy of Sciences USA 85, 2444-2448 (1988)) or Altschul et al.'s BLAST algorithm (Nature Genetics 6, 119-129 (1994)) and compared with the sequence entries available in publicly accessible databases. Publicly accessible nucleotide sequence databases are, for example, the European Molecular Biology Laboratory database (EMBL, Heidelberg, Germany) or the National Center for Biotechnology Information database (NCBI, Bethesda, MD, USA).

The novel DNA sequence responsible for replication of the plasmid pTET3, which sequence is provided by the present invention as SEQ ID NO:1, and which bears the repA gene responsible for replication and the parA gene responsible for stability, was obtained in this manner. The amino acid sequences of the encoded proteins were furthermore deduced from this DNA sequence. SEQ ID NO:2 shows the resultant amino acid sequence of the stabilisation protein ParA, while SEQ ID NO:3 shows the resultant amino acid sequence of the replication protein RepA of pTET3.

The novel DNA sequence responsible for replication of the plasmid pCRY4, which sequence is provided by the present invention as SEQ ID NO:4, and which bears the repA gene responsible for replication of pCRY4, was furthermore obtained in this manner. SEQ ID NO:5 shows the deduced

amino acid sequence of the replication protein RepA of plasmid pCRY4.

Few naturally occurring genes that impart resistance to antibiotics in *Corynebacterium glutamicum* are known. The inventors were accordingly all the more surprised to find that plasmid pTET3 imparts resistance to the antibiotics tetracycline, streptomycin, spectinomycin and sulfamethoxazole.

In order to identify antibiotic resistance genes on new plasmids, the strain to be investigated, in the present case *Corynebacterium glutamicum* LP-6, and a sensitive control strain, in the present case *Corynebacterium glutamicum* ATCC13032, are initially tested for resistance or sensitivity to various antibiotics and concentrations of antibiotics. The National Committee of Clinical Laboratory Standards (NCCLS) experimental procedure is preferably used for this purpose ("Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically", fourth edition; Approved Standard, M7-A4, NCCLS 17(2), (1997)). Using the method of "Approved Standard M7-A4", it is possible to determine inhibition concentrations and thus to ascertain the resistance of the investigated strain of bacteria.

The plasmid to be investigated, in the present case pTET3, is then isolated from strain LP-6 as described above and used to transform a suitable control or indicator strain, in the present case strain ATCC13032. Methods for transforming coryneform bacteria are described, for example, in Thierbach et al. (Applied and Environmental Microbiology 29, 356-362 (1988)), in Liebl et al. (FEMS Microbiology Letters 65, 299-304 (1989)) or in Dunican et al. (Bio/Technology 7, 1067-1070 (1989)). Selection is performed on conventional, complex nutrient media, such as

for example brain-heart bouillon or Luria-Bertani medium, which are supplemented with the appropriate antibiotics. The antibiotic and the concentration thereof for this selection process is determined on the basis of the above-mentioned "Approved Standard, M7-A4". In this manner, strain ATCC13032[pTET3], is obtained by selection for tetracycline resistance. The resistance/sensitivity of strain ATCC13032[pTET3] and of the control strain ATCC13032 is then investigated using the above-mentioned method, yielding the result that strain ATCC13032[pTET3] is resistant to the antibiotics tetracycline, streptomycin, spectinomycin and sulfamethoxazole.

This antibiotic resistance was further characterised by cloning and sequencing. To this end, plasmid pTET3 is isolated from strain LP-3 or ATCC13032[pTET3], treated with suitable restriction enzymes, mixed with cloning vectors treated in the same manner and treated with T4 DNA ligase. The ligation mixture is transferred by transformation into a suitable cloning host of Escherichia coli. Selection for transformants is performed on a complex nutrient medium, which is supplemented with the appropriate antibiotic. The person skilled in the art will find instructions relating to this method in Sambrook et al. Examples of suitable cloning vectors are pUC19 (Yanish-Perron et al., Gene 33, 103-119 (1985)), pK18mob2 (Tauch et al., Plasmid 40, 126-139 (1998)) or pCR2.1 (Invitrogen BV, Groningen, Netherlands). Suitable hosts are in particular those *E. coli* strains with restriction and recombination defects. One example of such a strain is the strain DH5αMCR, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87, 4645-4649 (1990)). Transformation methods are described, for example, in Hanahan (Journal of Molecular Biology 166, 577-580 (1983)) or Tauch et al. (Plasmid 40, 126-139 (1998)). Transformant

selection is performed by using the antibiotics to which plasmid pTET3 imparts resistance. The plasmid DNA of the resultant transformants is then isolated and the cloned DNA fragments of plasmid pTET3 are sequenced. The sequences are then analysed as described above and compared with databases of collected DNA sequences.

The inventors discovered in this manner that the genes which impart resistance to the antibiotics tetracycline, streptomycin, spectinomycin and sulfamethoxazole are located on a continuous DNA fragment. This DNA fragment is shown as a restriction map in Figure 5. The DNA portion containing the genes tetR, tetA and aadA is shown as a sequence in SEQ ID NO:6 and is provided by the invention.

The amino acid sequences of the protein encoded by the particular gene were furthermore deduced from the ascertained DNA sequence. SEQ ID NO:7 shows the deduced amino acid sequence of the tetracycline resistance protein TetA encoded by the tetA gene and SEQ ID NO:8 shows the deduced amino acid sequence of the spectinomycin/ streptomycin resistance protein AadA encoded by the aadA gene. SEQ ID NO:9 shows the coding region of the tetR gene and SEQ ID NO:10 the amino acid sequence of the tetracycline resistance repressor protein TetR.

Coding DNA sequences arising from SEQ ID NO:6 based on the degeneracy of the genetic code are also provided by the present invention. DNA sequences which hybridise with SEQ ID NO:1 or parts of SEQ ID NO:1 are similarly provided by the invention. Conservative substitutions of amino acids in proteins, for example the substitution of glycine for alanine or of aspartic acid for glutamic acid, are known to those of skill in the art as "sense mutations", which result in no fundamental change in activity of the protein, i.e. they are functionally neutral. Amino acid sequences

arising in a corresponding manner from SEQ ID NOS:7, 8 and 10 are also provided by the present invention.

The DNA fragments of plasmids pTET3 and pCRY4 from *Corynebacterium glutamicum* strain LP-6 may then be combined with DNA fragments of known plasmids of other microorganisms, such as for example *Escherichia coli* or *Corynebacterium glutamicum*, to yield further, novel plasmid vectors. For the purposes of the present invention, it is preferred to use plasmid DNA from other strains of the species *Corynebacterium glutamicum*. This approach, known as self cloning, has the advantage that no foreign nucleotide sequences are introduced in the species *Corynebacterium glutamicum*. Such further developed plasmid vectors may consist solely of constituents of the novel plasmid pTET3, i.e. of a replication region and at least one antibiotic resistance region, which is used as a selection marker. One example of such a vector is the plasmid vector pSELF3-1 shown in Figure 6. These vectors may, however, also be composed of constituents of a known plasmid and constituents of pTET3 or pCRY4. One example of such a vector is the plasmid vector pSELF1-1 shown in Figure 7, in which the known cryptic plasmid pGA1 (U.S. Pat. No. 5,175,108) has been provided with the tetA gene which imparts tetracycline resistance of pTET3.

The plasmid vectors constructed from the novel plasmids pTET3 and pCRY4 may advantageously be used for the fermentative production of industrially interesting metabolites such as amino acids, vitamins and nucleotides.

For example, within the framework of the present invention, a lysC(FBR) allele of *C. glutamicum* which encodes a feed-back resistant aspartate kinase was cloned into *C. glutamicum* ATCC13032 by means of pSELF1-1. In this manner,

a self-cloned lysine producing strain of *C. glutamicum* was produced.

By way of further example, the panD gene coding for aspartate α-decarboxylase from *C. glutamicum* was cloned into the *C. glutamicum* strain ATCC13032ΔilvA by means of pSELF1-1. In this manner, a self-cloned pantothenic acid producing strain of *C. glutamicum* was produced.

One very particular advantage of the novel plasmids pTET3 and pCRY4 and further plasmid vectors based thereon is that they exhibit an unusually high level of compatibility with known plasmids or plasmid vectors.

It was thus found that plasmid pTET3 may coexist in the presence of or is compatible with plasmid vectors based on pGA1 (U.S. Pat. No. 5,175,108), pAG3 (U.S. Pat. No. 5,158,891), pBL1 (Santamaria et al., Journal of General Microbiology 130, 2237-2246 (1984)) or on pHM1519 (Miwa et al., Agricultural and Biological Chemistry 48, 2901-2903 (1984)). This compatibility of pTET3 is still retained when the host cell concerned already contains two or more known plasmid vectors, for example a pBL1 derivative and simultaneously a pHM1519 derivative. pTET3's capacity to coexist with known plasmids or plasmid vectors is ensured over a sufficiently long period of time or for a sufficiently large number of generations.

It has furthermore been found that plasmid pCRY4 may coexist in the simultaneous presence of or is compatible with plasmids pTET3, pGA1 (U.S. Pat. No. 5,175,108) and pGA2 (U.S. Pat. No. 5,175,108) in the presence of plasmid vectors based on pAG3 (U.S. Pat. No. 5,158,891), pBL1 (Santamaria et al., Journal of General Microbiology 130, 2237-2246 (1984)) or on pHM1519 (Miwa et al., Agricultural and Biological Chemistry 48, 2901-2903 (1984)). This

compatibility of pCRY3 is still retained when the host cell concerned already contains two or more known plasmid vectors, for example a pBL1 derivative and simultaneously a pHM1519 derivative. pCRY4's capacity to coexist with known plasmids or plasmid vectors is ensured over a sufficiently long period of time or for a sufficiently large number of generations.

The improved compatibility of plasmids pTET3 and pCRY4 may advantageously be used for improving strains which produce amino acids, vitamins and nucleotides. Sahm and Eggeling (Applied and Environmental Microbiology 65, 1973-1979 (1999)) thus describe the pantothenic acid producing strain ATCC13032ΔilvA [pECM3ilvBNCD, pEKEx2panBC]. This strain bears the pHM1519 derivative pECM3ilvBNCD and the pBL1 derivative pEKEx2panBC. It proved possible to achieve a distinct improvement in the performance characteristics of the stated strain, which already contains two plasmids, after transferring the panD gene by means of the plasmid vector pSELF3-1.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1: Restriction map of plasmid pTET3.
- Figure 2: Restriction map of plasmid pCRY4.
- Figure 3: Map of replication region of plasmid pTET3.
- Figure 4: Map of replication region of plasmid pCRY4
- Figure 5: Map of antibiotic resistance region of plasmid pTET3.
- Figure 6: Map of plasmid vector pSELF3-1.
- Figure 7: Map of plasmid vector pSELF1-1.

The lengths stated should be considered to be approximate. The abbreviations and terms used have the following meaning:

bps: Base pairs

AvrII: Restriction site for restriction enzyme AvrII

ClaI: Restriction site for restriction enzyme ClaI

EcoRI: Restriction site for restriction enzyme EcoRI

EcoRV: Restriction site for restriction enzyme EcoRV

FspI: Restriction site for restriction enzyme FspI

HindIII: Restriction site for restriction enzyme HindIII

HpaI: Restriction site for restriction enzyme HpaI

MunI: Restriction site for restriction enzyme MunI

NruI: Restriction site for restriction enzyme NruI

PstI: Restriction site for restriction enzyme PstI

SacI: Restriction site for restriction enzyme SacI

SacII: Restriction site for restriction enzyme SacII

SalI: Restriction site for restriction enzyme SalI

ScaI: Restriction site for restriction enzyme ScaI

SmaI: Restriction site for restriction enzyme SmaI

SpeI: Restriction site for restriction enzyme SpeI

SphI: Restriction site for restriction enzyme SphI

XbaI: Restriction site for restriction enzyme XbaI

XhoI: Restriction site for restriction enzyme XhoI

aadA: Gene for spectinomycin/streptomycin resistance protein

parA: Gene for stabilisation protein ParA

sulI: Gene for the sulfamethoxazole resistance protein

repA: Gene for the replication protein RepA

tetA: Gene for the tetracycline resistance protein

tetR: Gene for the tetracycline repressor protein

DETAILED DESCRIPTION OF THE INVENTION

The present invention is illustrated in greater detail by the following practical examples.

The following strains of bacteria were used:

Corynebacterium glutamicum LP-6 was deposited in the context of EP-B 0 472 869 with Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) under number DSM5816. The storage period for

DSM5816 has been extended pursuant to rule 9.1 of the Budapest Treaty. DSM5816 has the following taxonomic features:

- Cell shape: Y-shaped branching
- Peptidoglycan: meso-diaminopimelic acid
- Mycolic acids: Corynebacterium mycolic acids with a high level of similarity to DSM20300
- Fatty acid pattern: fatty acid pattern typical of Corynebacterium with unbranched, saturated and unsaturated fatty acids with a high level of similarity to DSM20300.
- G+C content: 55.1%
- 16S rDNA sequence: 98.6% identical in comparison with DSM20300
- DNA-DNA homology: 81.6% to DSM20300

Corynebacterium glutamicum ATCC13032 was obtained from the American Type Culture Collection (Manassas, USA).

Corynebacterium glutamicum ATCC13032ΔilvA is deposited with Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) under number DSM12455.

The general genetic methods stated and the nutrient media used in the following Examples are described in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Electrically-assisted transfer of plasmid DNA was performed using the method of Liebl et al. (FEMS Microbiology Letters 65, 299-304 (1989)).

The DNA fragments described in the following Examples were sequenced in accordance with the dideoxy chain termination method according to Sanger et al. (Proceedings of the National Academy of Sciences USA 74, 5463-5467 (1977)). The resultant raw sequence data were processed using the

"STADEN software package" (Staden, Molecular Biotechnology 5, 233-241 (1996)). Computer-aided coding range analysis was performed using XNIP software (Staden, Molecular Biotechnology 5, 233-241 (1996)). Further sequence analysis was performed using the "BLAST programs" (Altschul et al., Nucleic Acids Research 25, 3389-3402 (1997)).

Example 1

Isolation and characterisation of the novel plasmids pTET3 and pCRY4

In order to identify novel plasmids and isolate plasmid DNA, the bacterial strain *Corynebacterium glutamicum* LP-6 was cultured in LB medium and isolated in accordance with the instructions given in "NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)" (Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). The isolated plasmid DNA was separated in a 0.8% agarose gel and the plasmid bands corresponding to the novel plasmids pTET3 and pCRY4 were each reisolated separately from the agarose gel. The experimental procedure was in accordance with "QIAEX II Handbook for DNA Extraction from Agarose Gels" (Qiagen GmbH, Hilden, Germany, 1997). The reisolated plasmid DNA of pTET3 was then digested in accordance with the manufacturers' instructions with the restriction enzymes AvrII, MunI (New England Biolabs GmbH, Schwalbach, Germany), HpaI, ScaI, XbaI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) and SpeI (Roche Diagnostics GmbH, Mannheim, Germany) in each case individually and in combination. The restriction batches were then separated in a 0.8% agarose gel. By comparing the resultant DNA fragments with DNA fragments of known length (DNA Molecular Weight Marker X, Roche Diagnostics GmbH, Mannheim, Germany), the restriction map of plasmid pTET3 from

Corynebacterium glutamicum LP-6 shown in Figure 1 was determined.

The reisolated plasmid DNA of the novel plasmid pCRY4 from *Corynebacterium glutamicum* LP-6 was then digested in accordance with the manufacturers' instructions with the restriction enzymes AvrII (New England Biolabs GmbH, Schwalbach, Germany), EcoRV, HpaI and ClaI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) in each case individually and in combination. The restriction batches were then separated in a 0.8% agarose gel. By comparing the resultant DNA fragments with DNA fragments of known length (DNA Molecular Weight Marker X, Roche Diagnostics GmbH, Mannheim, Germany), the restriction map of plasmid pCRY4 from *Corynebacterium glutamicum* LP-6 shown in Figure 2 was determined.

Example 2

Isolation and sequencing of the replication region of plasmid pTET3

In order to isolate a DNA region which is required for stable replication of the novel plasmids in coryneform bacteria, plasmid DNA was initially isolated from *Corynebacterium glutamicum* LP-6 by alkaline treatment of the bacterial cells. The experimental method is described in detail in the instructions for "NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)" (Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). The resultant DNA preparation of *Corynebacterium glutamicum* LP-6 was then separated in a 0.8% agarose gel and investigated for the presence of plasmid bands. The identified plasmid bands from *Corynebacterium glutamicum* LP-6 were assigned to the known plasmids pGA1 and pGA2 (U.S. Pat. No. 5,175,108) and the novel plasmids pTET3 and pCRY4. The plasmid bands corresponding to the plasmid pTET3 were reisolated from the agarose gel (c.f. Example 1). The experimental procedure may be found in "QIAEX II Handbook for DNA Extraction from Agarose Gels" (Qiagen GmbH, Hilden, Germany, 1997). The reisolated plasmid DNA was then digested with the restriction enzymes AvrII (New England Biolabs GmbH, Schwalbach, Germany) and HpaI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) and cloned into the vector pK18mob2 (Tauch et al., Plasmid 40, 126-139 (1998)) which had been cut with the restriction enzymes XbaI and SmaI (Pharmacia Biotech Europe GmbH, Freiburg, Germany). DNA restriction and DNA ligation using the enzyme T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany) were performed in accordance with the manufacturer's instructions. This ligation mixture was then electroporated into strain *Corynebacterium glutamicum* ATCC13032. Selection

was performed on LB agar containing 25 µg/ml of kanamycin. After 48 hours' incubation at 30°C, colonies were isolated which contained plasmids. The presence of plasmids in the transformed bacterial cells was shown using an alkaline lysis method in accordance with the instructions in "QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit" (Qiagen GmbH, Hilden, Germany, 1997). The isolated plasmid was named pTET3-Rep. Restriction analysis of pTET3-Rep and a comparison of the fragment lengths obtained with DNA fragments of known length (DNA Molecular Weight Marker X, Roche Diagnostics GmbH, Mannheim, Germany) revealed that pTET3-Rep consists of the cloning vector pK18mob2, which contains a DNA fragment from pTET3 of an approximate size of 4500 base pairs (bp).

For the purposes of double-stranded DNA sequencing of the approximately 4500 bp DNA fragment from pTET3-Rep, the DNA was isolated in accordance with the instructions of "NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)" (Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). Sequencing and subsequent coding region analysis revealed two open reading frames (ORFs) on the sequenced DNA fragment. Figure 3 shows a restriction map of the sequenced DNA fragment of pTET3-Rep, which also indicates the position of the two identified ORFs. Analysis with the BLAST programs revealed that ORF1 encodes a stabilisation protein designated as ParA and that ORF2 encodes a replication protein designated as RepA. ORF1 was accordingly designated as the parA gene and ORF2 as the repA gene. The DNA sequence of the cloned fragment is set forth in SEQ ID NO:1. The amino acid sequence of the stabilisation protein ParA, deduced from the DNA sequence, is set forth in SEQ ID no. 2 and the deduced amino acid sequence of the replication protein RepA is set forth in SEQ ID NO:3.

Example 3

Determination of the copy number of the pTET3 replicon in *Corynebacterium glutamicum* ATCC13032

In order to determine the copy number of plasmid pTET3-Rep, the bacterial strain *Corynebacterium glutamicum* ATCC13032 [pTET3-Rep] was cultured for 20 hours at 30°C in 100 ml of LB medium with 25 µg/ml of kanamycin. The total DNA of the strain was then isolated from 25 ml of bacterial culture using the method according to Tauch et al. (Plasmid 34, 119-131 (1995)). The resultant DNA was treated for 20 minutes at 37°C with 20 µg/ml of RNase/DNase-free (Roche Diagnostics GmbH, Mannheim, Germany) and, after phenol extraction, separated electrophoretically in 0.8% agarose gel. The agarose gel stained with ethidium bromide was photographed under UV light with a Cybertech CS1 camera system (Cybertech GmbH, Berlin, Germany) and the negative image was digitised with an HP Scanjet 6100 C/T Optical Scanner (Hewlett-Packard Co., Palo Alto, CA, USA). The band density of the DNA was quantified densitometrically using the Wincam computer system from Cybertech GmbH (Berlin, Germany). The copy number was calculated in accordance with the method of Miwa et al. (Agricultural and Biological Chemistry 48, 2901-2903 (1984)) assuming a chromosome size of 3082 kb (Bathe et al., Molecular and General Genetics 252, 255-265 (1996)) and revealed a value of 15 plasmids per chromosome for plasmid pTET3-Rep in *Corynebacterium glutamicum* ATCC13032.

Example 4

Isolation and sequencing of the replication region of plasmid pCRY4

In order to isolate the DNA region which is required for stable replication of the novel plasmid pCRY4 in coryneform bacteria, plasmid DNA was initially isolated from *Corynebacterium glutamicum* LP-6 by alkaline treatment of the bacterial cells. The experimental method may be found in the instructions for "NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)" (Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). The resultant DNA preparation of *Corynebacterium glutamicum* LP-6 was then separated in a 0.8% agarose gel and investigated for the presence of a pCRY4 plasmid band. The identified plasmid band corresponding to the novel plasmid pCRY4 was then reisolated from the agarose gel (c.f. Example 1). The experimental procedure may be found in "QIAEX II Handbook for DNA Extraction from Agarose Gels" (Qiagen GmbH, Hilden, Germany, 1997). The reisolated plasmid DNA was then digested with the restriction enzyme SphI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) and cloned into the vector pK18mob2 (Tauch et al., Plasmid 40, 126-139 (1998)) which had been cut with the restriction enzyme SphI. DNA restriction and DNA ligation using the enzyme T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany) were performed in accordance with the manufacturer's instructions. The ligation mixture was then transferred with electrical assistance into the coryneform bacterial strain *Corynebacterium glutamicum* ATCC13032. Selection was performed on LB agar containing 25 µg/ml of kanamycin. After 48 hours' incubation at 30°C, colonies containing plasmids were isolated. The presence of plasmids in the transformed bacterial cells was demonstrated by an alkaline lysis method in accordance with the instructions in "QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit" (Qiagen GmbH, Hilden, Germany, 1997). The isolated plasmid was named pCRY4-Rep. Restriction analysis of pCRY4-Rep and a comparison of the fragment lengths obtained with DNA

fragments of known length (DNA Molecular Weight Marker X, Roche Diagnostics GmbH, Mannheim, Germany) revealed that pCRY4-Rep contains an approximately 1900 bp DNA fragment.

For the purposes of double-stranded DNA sequencing of the approximately 1900 bp DNA fragment from pCRY4-Rep, the DNA was isolated in accordance with the instructions of "NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)" (Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). DNA sequencing and computer-aided coding region analysis allowed an open reading frame (ORF1) to be identified on the sequenced DNA fragment. Figure 4 shows the restriction map of the sequenced DNA fragment of pCRY4-Rep, which also indicates the position of the identified ORF. Analysis with the BLAST programs revealed that ORF1 encodes a replication protein (RepA), which was designated as the repA gene. The DNA sequence of the cloned fragment is reproduced as SEQ ID NO:4, while the deduced amino acid sequence of the replication protein RepA is shown in SEQ ID NO:5.

Example 5

Determination of the copy number of the pCRY4 replicon in *Corynebacterium glutamicum* ATCC13032

In order to determine the copy number of plasmid pCRY4-Rep, the bacterial strain *Corynebacterium glutamicum* ATCC13032 [pCRY4-Rep] was cultured for 20 hours at 30°C in 100 ml of LB medium with 25 µg/ml of kanamycin. The total DNA of the strain was then isolated from 25 ml of bacterial culture using the method according to Tauch et al. (Plasmid 34, 119-131 (1995)). The resultant DNA was treated for 20 minutes at 37°C with 20 µg/ml of RNase/DNase-free (Roche Diagnostics GmbH, Mannheim, Germany) and, after phenol extraction, separated electrophoretically in 0.8% agarose

gel. The agarose gel stained with ethidium bromide was photographed under UV light with a Cybertech CS1 camera system (Cybertech GmbH, Berlin, Germany) and the negative image was digitised with an HP Scanjet 6100 C/T Optical Scanner (Hewlett-Packard Co., Palo Alto, CA, USA). The band density of the DNA was quantified densitometrically using the Wincam computer system from Cybertech GmbH (Berlin, Germany). The copy number was calculated in accordance with the method of Miwa et al. (Agricultural and Biological Chemistry 48, 2901-2903 (1984)) assuming a chromosome size of 3082 kb (Bathe et al., Molecular and General Genetics 252, 255-265 (1996)) and revealed a value of 3 plasmids per chromosome for plasmid pCRY4-Rep in *Corynebacterium glutamicum* ATCC13032.

Example 6

Isolation and sequencing of the antibiotic resistance region of plasmid pTET3

In order to identify antibiotic resistance regions on the novel plasmids pTET3 or pCRY4, the resistant test strain *Corynebacterium glutamicum* LP-6 and the sensitive control strain *Corynebacterium glutamicum* ATCC13032 were initially cultured in the presence and absence of various antibiotics and antibiotic concentrations in accordance with the experimental method of the National Committee of Clinical Laboratory Standards (National Committee of Clinical Laboratory Standards, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard, M7-A4 (1997)). The antibiotics required for this test, *inter alia* the antibiotics tetracycline, spectinomycin, streptomycin and sulfamethoxazole, were obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany) and used in the concentrations stated in "Approved Standard M7-A4". The nutrient medium required for this

test, "MÜLLER-HINTON bouillon" was obtained from Merck KGaA (Darmstadt, Germany) and used in accordance with the manufacturer's instructions. Using the method of "Approved Standard M7-A4", it is possible to determine inhibition concentrations (Table 1) and to identify the resistance of the bacterial strain *Corynebacterium glutamicum* LP-6 to the antibiotics tetracycline, spectinomycin, streptomycin and sulfamethoxazole. Plasmid DNA isolated from *Corynebacterium glutamicum* LP-6 using an alkaline lysis method ("NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)", Clonetech Laboratories GmbH, Heidelberg, Germany, 1997) was then transferred with electrical assistance into *Corynebacterium glutamicum* ATCC13032. Selection was performed directly for the presence of the identified tetracycline resistance in the primary selection on LB agar containing 5 µg/ml of tetracycline. The presence of a plasmid in the transformed bacterial strain *Corynebacterium glutamicum* ATCC13032 was then demonstrated by an alkaline lysis method ("NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)", Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). Restriction analysis of the isolated plasmid DNA and comparison of the resultant fragment lengths with DNA fragments of known length (DNA Molecular Weight Marker X, Roche Diagnostics GmbH, Mannheim, Germany) and with DNA fragments of plasmid pTET3 revealed that the transformed plasmid which imparts tetracycline resistance is the plasmid pTET3. The transformed strain was named *Corynebacterium glutamicum* ATCC13032 [pTET3].

Another resistance test with the isolated, resistant test strain *Corynebacterium glutamicum* ATCC13032 [pTET3] and the sensitive control strain *Corynebacterium glutamicum* ATCC13032 in accordance with the instructions of the National Committee of Clinical Laboratory Standards in the

presence of various concentrations of the antibiotics tetracycline, spectinomycin, streptomycin and sulfamethoxazole demonstrated that the test strain *Corynebacterium glutamicum* ATCC13032 [pTET3] is resistant to these antibiotics (Table 1).

Table 1

Minimum inhibition concentration (µg of antibiotic per ml) of various *Corynebacterium glutamicum* strains

Antibiotic	ATCC13032	LP-6	ATCC13032 [pTET3]
Tetracycline	≤ 0.75	≤ 12	≤ 12
Spectinomycin	≤ 50	> 2000	> 2000
Streptomycin	≤ 0.5	≤ 100	≤ 100
Sulfamethoxazole	≤ 150	≤ 300	≤ 300

The symbols are defined as follows:

> : The minimum inhibition concentration is greater than the stated value.

≤ : The minimum inhibition concentration is less than or equal to the stated value.

The antibiotic resistance of pTET3 was further characterised by reisolating the plasmid DNA from *Corynebacterium glutamicum* ATCC13032 [pTET3] using an alkaline lysis method ("NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)",

Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). The plasmid DNA was then cleaved with the restriction enzymes HindIII or SacI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) and ligated into the *Escherichia coli* cloning vectors pK18mob2 (Tauch et al., Plasmid 40, 126-139 (1998)) or pUV19 (Pharmacia Biotech Europe GmbH, Freiburg, Germany). DNA restriction and DNA ligation using the enzyme T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany) were performed in accordance with the manufacturer's instructions. The ligation batch was then electroporated into the bacterial strain *Escherichia coli* DH5αMCR (Tauch et al., FEMS Microbiology Letters 123, 343-348 (1994)). After selection on LB agar containing 5 µg/ml of tetracycline or 250 µg/ml of spectinomycin, transformed colonies were obtained, the plasmid vectors of which contained sections of DNA from plasmid pTET3. The presence of plasmids vectors was proven by an alkaline lysis method ("QIAGEN Plasmid Miniprep Handbook for Plasmid DNA", Qiagen GmbH, Hilden, Germany, 1997). Restriction analysis of the isolated plasmid DNA and comparison of the resultant fragment lengths with DNA fragments of known length revealed that the isolated plasmid named pTET3-H9 consists of the plasmid vector pK18mob2 and an approximately 4000 bp DNA fragment from pTET3, and that the isolated plasmid named pXCS10 consists of the plasmid vector pUC19 (Pharmacia Biotech Europe GmbH, Freiburg, Germany) and an approximately 6750 bp DNA fragment from pTET3. The plasmid vector pTET3-H9 obtained from cloning with the restriction enzyme HindIII, imparts tetracycline resistance (5 µg/ml) in *Escherichia coli* DH5αMCR, while the plasmid vector pXCS10 obtained from cloning with the restriction enzyme SacI imparts resistance to the antibiotics spectinomycin (250 µg/ml), streptomycin (250 µg/ml) and sulfamethoxazole (300 µg/ml). A comparison of the restriction analyses of

the cloned DNA fragments of pTET3 in plasmid vectors pTET3-H9 and pXCS10 moreover demonstrated that both DNA fragments overlap by approximately 2400 bp and may thus be combined into a continuous DNA strand of a length of approximately 8350 bp.

For the purposes of double-stranded DNA sequencing of a continuous, approximately 7300 bp DNA fragment from pTET3 which imparts resistance to tetracycline, spectinomycin and streptomycin, DNA was isolated from plasmids pTET3-H9 and pXCS10 in accordance with the instructions of "QIAprep Miniprep Handbook for Purification of Plasmid DNA" (Qiagen GmbH, Hilden, Germany, 1997). After sequencing and sequence analysis, four open reading frames (ORFs) could be determined on the sequenced DNA fragment. Figure 5 shows a restriction map of the sequenced DNA region of pTET3 and the position of the identified open reading frames (ORFs). Analysis revealed that ORF1 represents a tetR gene which encodes a tetracycline resistance repressor protein (TetR), ORF2 represents a tetA gene which encodes a tetracycline resistance protein (TetA), ORF3 represents an aadA gene which encodes a spectinomycin/streptomycin resistance protein (AadA) and ORF4 represents a sulI gene which encodes a sulfamethoxazole resistance protein (SulI). The DNA sequence of the resistance region of pTET3 is reproduced in SEQ ID NO:6. The amino acid sequence of the tetracycline resistance protein (TetA), deduced from the sequence data, is shown in SEQ ID NO:7 and the amino acid sequence of the spectinomycin/streptomycin resistance protein (AadA), deduced from the sequence data, is shown in SEQ ID NO:8. The coding region of the tetR gene which encodes the tetracycline resistance repressor protein (TetR) is also shown in SEQ ID NO:9 and the deduced amino acid sequence in SEQ ID NO:10.

Example 7

Coexistence of plasmid pTET3 with known coryneform plasmids in *Corynebacterium glutamicum* ATCC13032

The bacterial strain *Corynebacterium glutamicum* ATCC13032 [pTET3] produced in Example 6 was used to analyse the coexistence of the novel plasmid pTET3 from *Corynebacterium glutamicum* LP-6 with known coryneform plasmids.

Electrocompetent cells of this strain were produced, into which plasmid vectors consisting of known plasmids of coryneform bacteria and selection marker fractions were transferred. Plasmid vectors pGA1-KE12, pAG3-Xba, pEBM2 (Tauch et al., Archives of Microbiology 169, 303-312 (1998)), pECM2 (Tauch et al., FEMS Microbiology Letters 123, 343-348 (1994)) and pECM3 were selected for this DNA transfer. Plasmid pGA1-KE12 is an EcoRI fusion of the cryptic plasmid pGA1 from *Corynebacterium glutamicum* LP-6 with vector pK18mob2 (Tauch et al., Plasmid 40, 126-139 (1998)). Plasmid pAG3-Xba is an XbaI fusion of pAG3 and pK18mob2. Plasmid pECM3 is a BamHI-BgIII deletion of pECM2. Once transfer of the plasmid vectors pGA1-KE12 (pGA1 derivative), pAG3-Xba (pAG3 derivative), pEBM2 (pBL1 derivative) and pECM2 (pHM1519 derivative), which impart kanamycin resistance, was complete, selection was performed on LB agar containing 25 µg/ml of kanamycin. Plasmid pECM3, a pHM1519 derivative, which imparts chloramphenicol resistance, was additionally transferred into the resultant bacterial strain *Corynebacterium glutamicum* ATCC13032 [pTET3, pEBM2], which bears the plasmids pTET3 and pEBM2. After DNA transfer, selection was performed on LB agar containing 7.5 µg/ml of chloramphenicol (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). In order to confirm completion of the plasmid transfer, plasmid DNA was isolated from the resultant strains or transformants

("NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)", Clonetech Laboratories GmbH, Heidelberg, Germany, 1997) and detected in 0.8% agarose gel.

In this manner, the following strains of *Corynebacterium glutamicum* were produced:

- ATCC13032 [pTET3, pGA1-KE12]
- ATCC13032 [pTET3, pAG3-Xba]
- ATCC13032 [pTET3, pEBM2]
- ATCC13032 [pTET3, pECM2]
- ATCC13032 [pTET3, pEBM2, pECM3].

In order to provide further evidence of the coexistence of the novel plasmid pTET3 with known plasmid vectors, the strains produced were initially cultured for 24 hours at 30°C in LB medium, which had been supplemented with the appropriate antibiotics (5 µg/ml of tetracycline, 25 µg/ml of kanamycin and 10 µg/ml of chloramphenicol). 1 ml portions of each of the cultures were then washed twice in antibiotic-free LB medium. Dilution series of the washed bacterial suspensions were prepared in LB medium and suspensions of 0.1 ml, which contained 10^4 cells, were transferred in each case onto 100 ml of antibiotic-free and antibiotic-containing LB medium. These cultures were again cultured at 30°C over approximately 25 generations and growth monitored by measuring optical density at a wavelength of 580 nm using a spectrophotometer (Pharmacia LKB Novaspec II, Pharmacia, Freiburg, Germany). The cultures were cultured at least up to an optical density of 8 (optical density of 1 corresponds to 4×10^8 cells per ml).

The plasmid DNA was then isolated from the cultures and separated in 0.8% agarose gel. The resultant plasmid bands were identical under both culture conditions, i.e. in the presence and absence of antibiotics, and each exhibited the presence of plasmid pTET3 and of the transformed plasmid vector, i.e. pGA1-KE12, pAG3-Xba, pEBM2, pECM2, and pEBM2 plus pECM3.

Example 8

Coexistence of plasmid pCRY4 with other coryneform plasmids in *Corynebacterium glutamicum* LP-6

Corynebacterium glutamicum LP-6, in which pCRY4 already coexists with plasmids pGA1, pGA2 and pTET3, was used to analyse the coexistence of plasmid pCRY4 with known coryneform plasmids.

Further plasmid vectors consisting of known coryneform plasmids and selection marker fractions were transferred into this bacterial strain. Plasmid vectors pAG3-Xba, pEBM2 (Tauch et al., Archives of Microbiology 169, 303-312 (1998)), pECM2 (Tauch et al., FEMS Microbiology Letters 123, 343-348 (1994)) and pECM3 were used for this DNA transfer. Plasmid pECM3 is a BamHI-BgIII deletion of pECM2. Transfer of the plasmid vectors pAG3-Xba (pAG3 derivative), pEBM2 (pBL1 derivatives) and pECM2 (pHM1519 derivative) was selected on LB agar containing 25 µg/ml of kanamycin. The plasmid pECM3, a pHM1519 derivative, which imparts chloramphenicol resistance was additionally transferred into the resultant bacterial strain *Corynebacterium glutamicum* LP-6 [pEBM2], which bears the plasmids pGA1, pGA2, pTET3, pCRY4 and pEBM2. After DNA transfer, selection was performed on LB agar containing 7.5 µg/ml of chloramphenicol. In order to confirm successful plasmid transfer, plasmid DNA was isolated from the resultant

strains or transformants ("NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)",Clonetech Laboratories GmbH, Heidelberg, Germany, 1997) and detected in 0.8% agarose gel.

In this manner, the following strains of *Corynebacterium glutamicum* were produced:

- LP-6 [pAG3-Xba]
- LP-6 [pEBM2]
- LP-6 [pECM2]
- LP-6 [pEBM2, pECM3].

(It should be noted that the recipient strain, Corynebacterium glutamicum LP-6, already contains plasmids pGA1, pGA2, pTET3 and pCRY4.)

In order to provide further evidence of the coexistence of the plasmid pCRY4 with known plasmid vectors, the strains produced were initially cultured for 24 hours at 30°C in LB medium, which had been supplemented with the appropriate antibiotics (5 µg/ml of tetracycline, 25 µg/ml of kanamycin and 10 µg/ml of chloramphenicol). 1 ml portions of the bacterial cultures were then washed twice in antibiotic-free LB medium. Dilution series of the washed bacterial suspensions were prepared in LB medium and suspensions of 0.1 ml, which contained 10^4 cells, were transferred in each case onto 100 ml of antibiotic-free and antibiotic-containing LB medium. These cultures were again cultured at 30°C over approximately 25 generations and growth monitored by measuring optical density at a wavelength of 580 nm using a spectrophotometer (Pharmacia LKB Novaspec II, Pharmacia, Freiburg, Germany). The cultures were cultured at least up to an optical density of 8 (optical density of

1 corresponds to 4×10^8 cells per ml). The plasmid DNA was then isolated from the cultures and separated in 0.8% agarose gel. The resultant plasmid bands were identical under selective and non-selective culture conditions, i.e. in the presence and absence of antibiotics, and each exhibited the presence of plasmids pGA1, pGA2, pTET3 and pCRY4 and of the transformed plasmid vector, i.e. pAG3-Xba, pEBM2, pECM2 and pEBM2 plus pECM3.

Example 9

Construction of plasmid vector pSELF3-1 from pTET3

In order to construct a plasmid vector consisting solely of components of the novel plasmid pTET3, the total plasmid DNA from *Corynebacterium glutamicum* LP-6 was isolated by alkaline treatment of the bacterial cells ("NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT3167-1)", Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). The resultant DNA preparation was then separated in a 0.8% agarose gel. The plasmid band corresponding to the novel plasmid pTET3 was reisolated from the agarose gel ("QIAEX II Handbook for DNA Extraction from Agarose Gels", Qiagen GmbH, Hilden, Germany). The reisolated plasmid DNA was then digested with the restriction enzyme XhoI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) in accordance with the manufacturer's instructions. The restriction batch was separated in a 0.8% agarose gel and an approximately 2500 bp DNA fragment, on which, according to DNA sequence data (Example 6), the tetracycline resistance region is located, was reisolated. The isolated pTET3 DNA was then cleaved with the restriction enzymes AvrII (New England Biolabs GmbH, Schwalbach, Germany) and HpaI (Pharmacia Biotech Europe GmbH, Freiburg, Germany). The cleavage batch was also separated in a 0.8% agarose gel and the approximately 4500

bp DNA fragment, on which, according to the DNA sequence information, the replication region of pTET3 is located, was reisolated. The projecting DNA ends of both the reisolated DNA fragments were then filled in with the enzyme Klenow polymerase. The fill-in reaction with the enzyme Klenow polymerase was performed in accordance with the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The filled in DNA fragments were then ligated together by the enzyme T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. The ligation mixture was transferred into *Corynebacterium glutamicum* ATCC13032 by electroporation. Selection was performed on LB agar containing 5 µg/ml of tetracycline. After 48 hours' incubation at 30°C, colonies were isolated which contain the novel plasmid vector. The presence of plasmid vector in the transformed bacterial cells was demonstrated using an alkaline lysis method ("QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit", Qiagen GmbH, Hilden, Germany, 1997). The isolated plasmid was named pSELF3-1. Restriction analyses of pSELF3-1 and a comparison of the fragment lengths obtained with DNA fragments of known length yielded the restriction map in Figure 6.

Due to this construction scheme, plasmid pSELF3-1 consists solely of DNA fragments of the novel plasmid pTET3 and thus of DNA which originates solely from *Corynebacterium glutamicum*.

Example 10

Construction of plasmid vector pSELF1-1

Plasmid vector pSELF1-1 was produced from known plasmid pGA1 (US-A 5,175,108) using the tetracycline resistance gene from pTET3 (c.f. Examples 1 and 6).

To this end, the total plasmid DNA of *Corynebacterium glutamicum* LP-6 was initially isolated by alkaline treatment of the bacterial cells ("NucleoBond Nucleic Acid Purification Kits and Cartridges User Manual (PT1997-6)", Clonetech Laboratories GmbH, Heidelberg, Germany, 1997). The resultant DNA preparation was separated in a 0.8% agarose gel. The plasmid bands corresponding to the known plasmid pGA1 and the novel plasmid pTET3 were reisolated from the agarose gel ("QIAEX II Handbook for DNA Extraction from Agarose Gels", Qiagen GmbH, Hilden, Germany). The isolated DNA from pGA1 was then cleaved with the restriction enzyme SalI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) in accordance with the manufacturer's instructions. The isolated plasmid DNA of pTET3 was cleaved with the restriction enzyme XhoI (Pharmacia Biotech Europe GmbH, Freiburg, Germany). The restriction batch of pTET3 was separated in a 0.8% agarose gel and an approximately 2500 bp DNA fragment, on which, according to DNA sequence data (Example 6), the tetracycline resistance region is located, was reisolated. The produced DNA fragment of pGA1 and the reisolated DNA fragment of pTET3 were then ligated together by means of T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. The ligation mixture was transferred into *Corynebacterium glutamicum* ATCC13032 by electroporation. Selection was performed on LB agar containing 5 µg/ml of tetracycline. After 48 hours' incubation at 30°C, colonies were isolated which contained the novel plasmid vector. The presence of plasmid vector in the transformed bacterial cells was proven by an alkaline lysis method ("QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit", Qiagen GmbH, Hilden, Germany, 1997). The isolated plasmid was named pSELF1-1. Restriction analyses of pSELF1-1 and a comparison of the fragment lengths obtained with DNA fragments of

known length yielded the restriction map which is attached as Figure 7.

Due to this construction method, plasmid pSELF1-1 consists solely of DNA fragments which originate solely from Corynebacterium glutamicum.

Example 11

Production of lysine using pSELF1-1

In order to increase the copy number of a gene which is involved in the biosynthesis of amino acid lysine in coryneform bacteria, the lysC(FBR) gene from Corynebacterium glutamicum was selected. The lysC(FBR) gene encodes a form of the enzyme aspartate kinase which is resistant to the antimetabolite S-(2-aminoethyl)cysteine and was in cloned form on the plasmid vector pJC30 (Cremer et al., Applied and Environmental Microbiology 57, 1746-1752 (1991)).

In order to clone the lysC(FBR) gene into the plasmid vector pSELF1-1 described in Example 10, plasmid DNA of pSELF1-1 and of pJC30 was cleaved with the restriction enzymes EcoRI and ScaI (Pharmacia Biotech Europe GmbH, Freiburg, Germany). The restriction batches were then ligated together with the enzyme T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany) and transformed into the bacterial strain *Corynebacterium glutamicum* ATCC13032. Selection was performed on LB agar containing 5 µg/ml of tetracycline. Plasmid DNA was reisolated from transformed colonies by an alkaline lysis method ("QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit", Qiagen GmbH, Hilden, Germany, 1997). By restriction analysis of this plasmid DNA and comparison with DNA fragments of known length, the

plasmid pSELF1-lysC was isolated, which consists of the plasmid vector pSELF1-1 and the lysC(FBR) gene region.

The plasmids pSELF-lysC and the control vector pSELF1-1 were transferred into the strain *Corynebacterium glutamicum* ATCC13032 by electroporation. Plasmid transfer was then proven by alkaline lysis and gel electrophoresis ("QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit", Qiagen GmbH, Hilden, Germany, 1997). The strains ATCC13032 [pSELF1-1] and ATCC13032 [pSELF1-lysC] constructed in this manner were used for the production of lysine.

Both strains were initially cultured for 24 hours at 30°C in 50 ml of Luria-Bertani medium containing 5 µg/ml of tetracycline. 1 ml portions of culture were then washed twice in mineral medium (Bröer et al., Applied and Environmental Microbiology 59, 316-321 (1993)), transferred into 100 ml of mineral medium with 5 µg/ml of tetracycline and incubated for a further 24 hours at 30°C. 5 ml portions of culture supernatant were pelletised for 15 minutes at 13800×g and 4°C and sterile-filtered with a Millex-GS filter unit (0.22 µm, Millipore S.A., Molsheim, France). Lysine was determined in the filtered culture supernatants by means of HPLC analysis using the method of Büntemeyer et al. (Cytotechnology 5, 57-67 (1991)). The resultant lysine concentrations after 24 hours' culturing are summarised in Table 2.

Table 2

Lysine concentration in culture supernatants of various strains of *Corynebacterium glutamicum*.

Host	Plasmid	Lysine concentration (g / l)
ATCC13032	pSELF1-1	0.02
ATCC13032	pSELF1-lysC	1.0

Example 12

Production of pantothenic acid using pSELF3-1

In order to increase the copy number of a gene which is involved in the biosynthesis of pantothenate in coryneform bacteria, the panD gene from *Corynebacterium glutamicum* ATCC13032 was selected. The panD gene encodes the enzyme L-aspartate α-decarboxylase and was in cloned form on the plasmid vector pND10 (Dusch et al., Applied and Environmental Microbiology 65, 1530-1539 (1999)).

In order to clone the panD gene into the novel plasmid vector pSELF3-1 described in Example 9, plasmid DNA of pSELF3-1 was cleaved with the restriction enzymes SacI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) and BstZ17I (New England Biolabs GmbH, Schwalbach, Germany) and plasmid DNA of pND10 was cleaved with the restriction enzymes SacI and ScaI (Pharmacia Biotech Europe GmbH, Freiburg, Germany) in accordance with the manufacturer's instructions. The restriction batches were then ligated together with the enzyme T4 DNA ligase in accordance with the manufacturer's instructions (Roche Diagnostics GmbH,

Mannheim, Germany) and transformed into the bacterial strain *Corynebacterium glutamicum* ATCC13032. Selection was performed on LB agar containing 5 µg/ml of tetracycline. Plasmid DNA was reisolated from the transformed colonies by alkaline lysis ("QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit", Qiagen GmbH, Hilden, Germany, 1997). By restriction analysis of the isolated plasmid DNA and comparison with DNA fragments of known length (DNA Molecular Weight Marker X, Roche Diagnostics GmbH, Mannheim, Germany), the plasmid pSELF3-panD was isolated, which consists of the plasmid vector pSELF3-1 and the region of pND10 which encodes the panD gene.

In order to analyse pantothenate production in coryneform bacteria, the constructed plasmid vector pSELF3-panD and the control vector pSELF3-1 were transferred into strain ATCC13032ΔilvA (Sahm et al., Applied and Environmental Microbiology 65, 1973-1979 (1999)). The presence of the plasmids was then proven by alkaline lysis ("QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit", Qiagen GmbH, Hilden, Germany, 1997). The strains ATCC13032ΔilvA [pSELF3-1] and ATCC13032ΔilvA [pSELF3-panD] constructed in this manner were used for the production of pantothenate.

The bacterial strains were initially cultured for 24 hours at 30°C in 50 ml of Luria-Bertani medium containing 5 µg/ml of tetracycline. 1 ml portions of the bacterial culture were then washed twice with CGXII medium (Keilhauer et al., Journal of Bacteriology 175, 5595-5603, (1993)), to which 2 mM of isoleucine (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) had been added, were transferred into 50 ml of CGXII medium with 2 mM of isoleucine and 5 µg/ml of tetracycline and cultured for 24 hours at 30°C. A further 50 ml of CGXII medium containing 2 mM of isoleucine were inoculated with 3 ml of this culture. After further

incubation of the batch for 24 hours at 30°C, 20 ml of the bacterial culture were pelletised for 10 minutes at 1250×g. The culture supernatant was then sterile-filtered with a Millex-GS filter unit (0.22 µm, Millipore S.A., Molsheim, France). Pantothenate concentration was determined in the filtered culture supernatants in accordance with the instructions in the Difco Manual, 10th Edition (Difco Laboratories, Detroit, Michigan, USA). The resultant pantothenate concentrations after 24 hours' culturing are summarised in Table 3.

Table 3

Pantothenate concentration in culture supernatants of various strains of *Corynebacterium glutamicum*.

Host	Plasmid	Pantothenate concentration (ng/ml)
ATCC13032ΔilvA	pSELF3-1	14.1
ATCC13032ΔilvA	pSELF3-panD	54.1

The constructed plasmid vector pSELF3-panD was also used further to improve strain ATCC13032ΔilvA [pEKEx2panBC, pECM3ilvBNCD] (Sahm et al., Applied and Environmental Microbiology 65, 1973-1979 (1999)). This strain already bears the genes ilvBNCD and panBC, which have an advantageous effect on pantothenate biosynthesis, on known plasmid vectors.

Plasmid vector pSELF3-panD and the control vector pSELF3-1 were transferred by electroporation into strain ATCC13032Δ ilvA [pEKEx2panBC, pECM3ilvBNCD] (Sahm et al., Applied and Environmental Microbiology 65, 1973-1979 (1999)). Selection

was performed on LB agar containing 5 µg/ml of tetracycline. The presence of the transferred plasmid vectors and the plasmids already present in the bacterial strain was then proven by alkaline lysis ("QIAGEN Plasmid Mini Handbook for Plasmid Mini Kit", Qiagen GmbH, Hilden, Germany, 1997). Both the strains constructed in this manner were also used in the manner described above for the production of pantothenate. The resultant pantothenate concentrations in the culture supernatants after 24 hours' culturing are shown in Table 4.

Table 4

Pantothenate concentration in culture supernatants of various strains of *Corynebacterium glutamicum*.

Host	Plasmids	Pantothenate concentration (ng/ml)
ATCC13032ΔilvA	pECM3ilvBNCD pEKEx2panBC pSELF3-1	18.3
ATCC13032ΔilvA	pECM3ilvBNCD pEKEx2panBC pSELF3-panD	655.2